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Antiviral Protection by Vesicular Stomatitis Virus-Specific Antibodies in Alpha/Beta Interferon Receptor-Deficient Mice

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The role of innate, alpha/beta interferon (IFN-α/β)-dependent protection versus specific antibody-mediated protection against vesicular stomatitis virus (VSV) was evaluated in IFN-α/β receptor-deficient mice (IFN-α/β $R^{0/0}$ mice). VSV is a close relative to rabies virus that causes neurological disease in mice. In contrast to normal mice, IFN-α/β $R^{0/0}$ mice were highly susceptible to infection with VSV because of ubiquitous high viral replication. Adoptive transfer experiments showed that neutralizing antibodies against the glycoprotein of VSV (VSV-G) protected these mice efficiently against systemic infection and against peripheral subcutaneous infection but protected only to a limited degree against intranasal infection with VSV. In contrast, VSV-specific T cells or antibodies specific for the nucleoprotein of VSV (VSV-N) were unable to protect IFN-α/β $R^{0/0}$ mice against VSV. These results demonstrate that mice are extremely sensitive to VSV if IFN-α/β is not functional and that under these conditions, neutralizing antibody responses mediate efficient protection, but apparently only against extraneuronal infection.

We studied the contribution of innate versus specific immunity in resistance against infection with vesicular stomatitis virus (VSV) by using alpha/beta interferon (IFN-α/β) receptor-deficient mice.

VSV, a close relative of rabies virus, is a cytopathic virus which elicits a strong humoral and cellular immune response in normal mice, resulting in clearance of the pathogen (14, 29). In such animals, neutralizing antibodies directed against the single envelope protein, the surface glycoprotein (VSV-G), are sufficient to control primary as well as secondary infection (16, 17). Studies with anti-IFN antibodies have shown that IFN-α/β is essential for natural resistance to VSV infection (10, 11). This was confirmed recently with mice lacking the receptor for IFN-α/β (IFN-α/β $R^{0/0}$) because of gene inactivation by homologous recombination in embryonic stem cells. These mice are highly susceptible to infection with cytopathic viruses such as VSV and Semliki Forest virus despite their ability to generate T-cell as well as neutralizing antibody responses within normal ranges (27). Infection of IFN-α/β $R^{0/0}$ mice with VSV leads to ubiquitous and nearly unrestricted viral replication, causing death within 3 to 6 days. The lethal dose for 50% of infected animals (LD_{50}) is less than 50 PFU in IFN-α/β $R^{0/0}$ mice, compared with about 10^8 PFU of VSV in C57BL/6 control animals after intravenous (i.v.) infection.

So far, studies of protective effector mechanisms against VSV have been performed in normal or immunodeficient mice, in which the virus usually replicates productively only in the central nervous system and cannot be found in organs of the periphery (24, 30, 32). The present study aimed at examining the protective role of neutralizing antibodies in mice devoid of interferon-mediated natural antiviral immunity. These studies show that in normal animals, primary infections with VSV are controlled by the concerted action of IFN-α/β and neutralizing antibodies; even when IFN-α/β cannot act, VSV-G-specific antibodies alone suffice to protect against secondary infection. In contrast, adoptively transferred VSV-G-specific memory B and T cells could not generate neutralizing antibodies quickly enough to mediate protection in IFN-α/β $R^{0/0}$ mice.

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MATERIALS AND METHODS

Mice. IFN-α/β $R^{0/0}$ mice with a homozygous inactivation of the type I (IFN-α/β) IFN receptor have been described recently (27). C57BL/6 (*H-2*^{b}), and 129Sv/Ev (*H-2*^{b}) mice were obtained from the breeding colony of the Institut für Zuchthygiene, Tierspital Zürich, Zürich, Switzerland. Mice were between 8 and 12 weeks of age.

Viruses. VSV Indiana (VSV-IND; Mudd-Summers isolate) had been originally obtained from D. Kolakofsky, University of Geneva, and was grown on BHK-21 cells infected at a low multiplicity of infection and plaqued on Vero cells (23). Recombinant vaccinia viruses expressing the glycoprotein and the nucleoprotein of VSV-IND (vacc-IND-G and vacc-IND-N, respectively) or the nucleoprotein of lymphocytic choriomeningitis virus (vacc-LCMV-N) have been described elsewhere (22). Vacc-IND-G and vacc-IND-N were a gift from B. Moss, Laboratory of Viral Diseases, National Institutes of Health, Bethesda, Md. Recombinant viruses were grown on BSC40 cells at a low multiplicity of infection and plaqued on the same cells.

Formalin inactivation of VSV. Inactivation of VSV-IND was performed as described previously (2). Briefly, formalin inactivation was performed at 4°C for 18 h at a formalin concentration of 0.0625% in minimal essential medium supplemented with 1% fetal calf serum. Only high-titer virus preparations were used for inactivation (10^8 to 10^9 PFU/ml), which could be diluted to reduce formalin concentrations after the inactivation procedure.

UV inactivation. A small volume (1 ml) of a VSV preparation (10^8 PFU/ml) was UV inactivated as a thin layer (<1 mm) in a petri dish for 5 min under a UV lamp (7UV; 15 W; Phillips) at a distance of 10 cm. Inactivation was controlled by a virus plaque assay (1, 2).

Serum neutralization test. Sera were prediluted 40-fold in supplemented minimal essential medium and heat inactivated for 30 min at 56°C. Serial twofold dilutions were mixed with equal volumes of virus diluted to contain 500 PFU/ml. The mixture was incubated for 90 min at 37°C in 5% CO_2. Then, 100 μl of the serum-virus mixture was transferred to Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. The monolayers were then overlaid with 100 μl of Dulbecco's modified Eagle's medium (DMEM) containing 1% methylcellulose. After incubation for 24 h at 37°C, the overlay was flicked off, and the monolayer was fixed and stained with 1% crystal violet. The highest dilution of serum that reduced the number of plaques by 50% was taken as the titer. To determine immunoglobulin G (IgG) titers, undiluted serum was pretreated with an equal volume of 0.1 M β-2-mercaptoethanol in saline (31).

Adoptive transfer. Single-cell suspensions of VSV-IND (2×10^6 PFU)- or

TABLE 1. Protection of IFN-α/β $R^{0/0}$ mice against infection with live VSV (2×10^6 PFU) by active immunization but not by transfer of VSV-immune spleen cells

Treatment	Anti-VSV neutralizing antibody titer[a] [$-\log_2$ (titer × 40)]	No. of mice dead on day 30 after infection/4 mice per group	VSV titer in brain (PFU/g)
None	ND	4[c]	1.5×10^5
VSV-IND[b] (2×10^7 PFU)			
UV inactivated (5 min)	6 ± 1.0	0	$<10^2$
Formalin inactivated	7 ± 1.5	1	$<10^2$
Live vacc-IND-G[b] (2×10^6 PFU)	8 ± 1.0	0	$<10^2$
Vacc-IND-G-immune spleen cells[d]	ND	4[e]	2×10^6
VSV-immune spleen cells[d]	ND	4[e]	2.5×10^5

[a] Sera for determination of neutralizing antibody titers (IgM and IgG) were taken on day 12 before infection. ND, not detectable.
[b] IFN-α/β $R^{0/0}$ mice were immunized i.v. with the indicated preparations 12 days before infection.
[c] All animals died on day 3.
[d] IFN-α/β $R^{0/0}$ mice were transfused with 3×10^7 spleen cells i.v. and challenged 12 h later with VSV.
[e] Animals died within 5 to 7 days after infection.

vacc-IND-G-primed mice (2×10^6 PFU) were prepared at day 8 and injected i.v. into irradiated (4.5 Gy) IFN-α/β $R^{0/0}$ mice. Donor cells were pooled from two to three individuals, and 3×10^7 cells were transferred per recipient.

VSV titer determination. The organs of VSV-infected animals were aseptically removed, weighed, and put in a vial with 2 ml of balanced salt solution. After grinding, the suspension of the organs was serially 10-fold diluted on ice, and 200 μl of each dilution was transferred to a confluent Vero cell monolayer (24-well plates) and incubated for 1 h at 37°C. The monolayers were then overlaid with 1% methylcellulose and incubated for 16 to 24 h at 37°C at 5% CO_2. The overlay was flicked off, and the cells were stained with 1% crystal violet. Titers were expressed as PFU per gram of organ.

Immunohistochemistry procedures. Frozen tissue section (5 μm thick) on slides were fixed in acetone for 10 min. Sections were sequentially stained with a rabbit antiserum specific for VSV (diluted 1:500), an alkaline phosphatase-labeled goat anti-rabbit IgG (Tago; diluted 8:80), and an alkaline phosphatase-labeled donkey anti-goat antibody (Jackson; diluted 1:80) and developed with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate by standard protocols.

RESULTS

Active immunization protects IFN-α/β $R^{0/0}$ from death caused by VSV. In a first experiment, IFN-α/β $R^{0/0}$ mice were immunized either with live vacc-IND-G (2×10^6 PFU) or with UV- (5 min) or formalin-inactivated VSV (2×10^7 PFU). Preliminary experiments had shown that mice lacking the IFN-α/β receptor survive infection with recombinant vacc-IND-G.

VSV-G-specific neutralizing antibody titers were determined 12 days after i.v. immunization. All three immunization protocols gave rise to comparable VSV-G-specific antibody titers. When mice were subsequently challenged with live VSV (2×10^6 PFU), IFN-α/β $R^{0/0}$ mice were protected from an otherwise lethal infection with VSV (Table 1). To test whether protection was antibody mediated or due to an antiviral action of activated T lymphocytes, IFN-α/β $R^{0/0}$ mice were reconstituted with 2×10^7 VSV- or vacc-IND-G-immune spleen cells (day 7) prior to infection with live VSV. Several experiments showed that recipients of immune spleen cells live 2 to 3 days longer than nonreconstituted IFN-α/β $R^{0/0}$ mice, but they were not protected and died by day 5 to 7 (Table 1).

Protection of IFN-α/β $R^{0/0}$ mice against systemic infection with VSV by VSV-G- but not VSV-N-specific immune serum. Next, we examined whether neutralizing antiserum specific for VSV-G or non-neutralizing antiserum with specificity for VSV-N alone is sufficient to protect IFN-α/β $R^{0/0}$ mice from systemic infection with VSV. IFN-α/β $R^{0/0}$ mice were reconstituted i.v. either with 100 μl of VSV-G (neutralizing titer, 1:58,000) or with VSV-N-specific immune serum (enzyme-linked immunosorbent assay [ELISA] titer, 1:4,500) prior to infection with VSV via the same route. Table 2 shows that five of six IFN-α/β $R^{0/0}$ mice transfused with VSV-G-specific serum survived infection with up to 2×10^7 PFU of VSV, which is close to the LD_{50} for normal C57BL/6 mice. Fifty days after infection, the serum of these mice still revealed a high neutralizing antibody titer (average titer, 1:2,560), suggesting endogenous generation of antibodies. These mice were resistant to secondary infection with VSV, and no live virus could be recovered from different organs 10 days after primary or secondary infection (data not shown).

In contrast, IFN-α/β $R^{0/0}$ animals that had been transfused with VSV-N-specific antiserum exhibited the same susceptibility to VSV infection as control animals and died by day 4 (Table 2).

Protection from local infection with VSV by VSV-G-specific immune serum. Although rhabdoviruses may be transmitted via insect or animal bites, VSV may also infect hosts via mucosal surfaces (32). Intranasal (i.n.) infection with VSV has been studied in immunocompetent as well as in immunodeficient mice; it was demonstrated that this route of infection results in rapid spread of viral antigen in distinct brain areas, with accompanying neuropathology (12, 13). Therefore, we compared the protective capacity of neutralizing antibodies after local infection with VSV. IFN-α/β $R^{0/0}$ mice were first reconstituted i.v. with VSV-G-specific immune serum and then infected with 2×10^5 PFU of VSV either i.n. or subcutaneously (s.c.) or into the footpad (i.f.). Protection of IFN-α/β $R^{0/0}$ mice by serum transfer against s.c. or i.f. infection with VSV was efficient (70 to 80%) although not complete. However, i.n. infection with 2×10^5 PFU of VSV was fatal for 60% of the immune serum-reconstituted IFN-α/β $R^{0/0}$ mice by day

TABLE 2. Protection against systemic infection of IFN-α/β $R^{0/0}$ mice with VSV-G- but not VSV-N-specific immune serum[a]

Antiserum treatment	Inoculum (PFU)	No. of mice dead/6 per group on day after infection:		
		4	8	30
None	$<3 \times 10^1$	0	0	1
	2×10^2	2	5	6
	2×10^6	6		
Anti-VSV-G	2×10^6	0	0	0
	2×10^7	0	1	1
Anti-VSV-N	2×10^4	5	6	
	2×10^6	6		

[a] Experiments were performed twice with similar results. IFN-α/β $R^{0/0}$ mice revealed VSV neutralizing titers of 7 to 8 [$-\log_2$ (titer × 40)] and VSV-N-specific ELISA titers of 1:600 to 1:800, as determined 1 h after passive transfer of immune serum. IFN-α/β $R^{0/0}$ mice were challenged i.v. 1 to 2 h after passive serum transfer.

TABLE 3. Protection of IFN-α/β $R^{0/0}$ mice against peripheral infection with VSV-IND[a]

Serum transfused	Route of challenge	No. of mice dead/10 per group on day after infection:		
		3	5	15
No	i.f.	2	8	10
Yes	i.f.	0	1	2
No	i.n.	7	10	
Yes	i.n.	2	6	8
No	s.c.	4	9	10
Yes	s.c.	0	2	3

[a] Data represent results for one of two independent experiments. IFN-α/β $R^{0/0}$ mice revealed VSV neutralizing antibody titers of 7 to 8 [$-\log_2$ (titer × 40)], as determined 6 h after passive transfer of serum. IFN-α/β $R^{0/0}$ mice were challenged with 2×10^5 PFU of live VSV 6 h after serum transfer.

5 and resulted in 80% mortality by day 15. Thus, the potential of antibodies to protect from infection via mucosal surfaces was very limited (Table 3).

Titers and viral spread in i.n. infected (2×10^5 PFU) IFN-α/β $R^{0/0}$ mice that had or had not been treated with immune serum were compared. Organs were removed at the time when about 50% of the mice had died (untreated, day 2; serum treated, day 4) and tested by a virus plaque assay. Untreated IFN-α/β $R^{0/0}$ mice revealed high titers of VSV on day 2, whereas no virus could be detected in three of five serum-reconstituted animals on day 4 (Fig. 1). The remaining two reconstituted mice were moribund and showed high titers of VSV in the brain, spinal cord, and lung but low titers in the spleen and liver (Fig. 1). Furthermore, immunohistochemical analysis of tissues from i.n. infected IFN-α/β $R^{0/0}$ mice confirmed the ubiquitous spread of VSV on day 2 in untreated animals. In accordance with the viral titers in Fig. 1, no (liver) or only small amounts (spleen) of viral antigen were detected on day 4 in animals that had been transfused with VSV immune serum prior to i.n. infection (Fig. 2). These results suggest that VSV is well controlled in organs accessible to antibodies, whereas replication in the brain is only poorly influenced by transferred specific antibodies.

Time dependence of protection by passive immunization with antibodies. Protection against lethal viral infection is normally achieved by active immunization before infection may occur. In some cases, particularly after rabies virus infection, postexposure vaccination is attempted and has been shown to be variably successful (reviewed in reference 6).

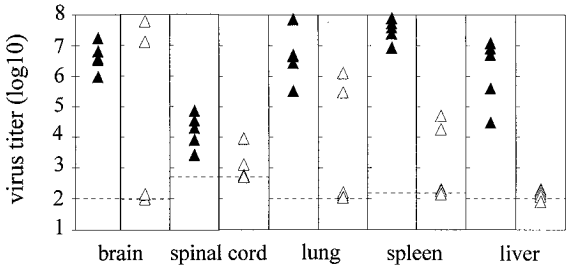

FIG. 1. Partial reduction of VSV titers in organs of IFN-α/β $R^{0/0}$ mice by VSV-G-specific immune serum after i.n. infection. IFN-α/β $R^{0/0}$ mice were left untreated (solid symbols) or reconstituted with 100 μl of VSV-G-specific immune serum (open symbols) 12 h before nasal challenge with 2×10^5, pfu of VSV. Organs of untreated mice were removed, and VSV titers were determined at day 2 after infection and compared with organs and titers of serum-reconstituted mice at day 4. Symbols represent titers for individual animals, expressed as $\log_{10}$ PFU per gram of organ. Dashed lines, detection levels.

Therefore, the protective capacity of immune serum adoptively transferred after infection with VSV was evaluated in IFN-α/β $R^{0/0}$ mice. Animals were first infected i.v. or i.n. with VSV, and 3 or 16 h later, 100 μl of immune serum was transferred. Passive immunization 3 h after systemic infection with 2×10^4 PFU of VSV conferred full protection; considerable protection (80%) was observed in mice inoculated i.v. with 2×10^6 VSV (Fig. 3A). In contrast, adoptive transfer of immune serum 16 h after infection was ineffective (Fig. 3C). Such animals exhibited high levels of viral replication in all organs tested (data not shown). The efficiency of passive postexposure immunization was also tested after i.n. challenge. In contrast to systemic infection, serum inoculation 3 and 16 h after i.n. infection had no or only marginal protective effects (Fig. 3B and D).

DISCUSSION

The present study demonstrates that neutralizing VSV-G-specific antibodies protected highly susceptible IFN-α/β $R^{0/0}$ mice from systemic and peripheral subcutaneous infection but did not exert significant protection against i.n. infection with VSV. In contrast, antibodies specific for the internal VSV-N as well as activated VSV-specific T and B lymphocytes did not confer significant protection in this model.

Several studies have shown that VSV-G-specific neutralizing antibodies are antivirally protective (9, 14); however, they could not exclude other synergistic mechanisms. Protection studies have so far been limited to the central nervous system, which is not readily accessible to antibodies (24, 30). This limitation of infection to the central nervous system was not seen in IFN-α/β $R^{0/0}$ mice. Despite their ability to generate normal humoral as well as cellular immune responses (27), they died of VSV infection within a few days; this documents the enormous importance of IFN-α/β in resistance against VSV.

It has been shown earlier that the specific IgG response in addition to IFN-α/β is necessary for protection against VSV (5, 18). Our experiments demonstrated that neutralizing antibodies protect mice efficiently even in the absence of IFN-α/β-mediated resistance, emphasizing the protective capacity of such antibodies. This model infection in mice is quite comparable to rabies infection in humans with respect to some of the neurological consequences; this result suggests that neutralizing antibodies are of prime importance in prevention or control of rhabdovirus infection. The finding that transfer of VSV-immune serum 16 h after infection fails to protect against VSV demonstrates the rapid evasion of VSV to the nervous system, where accessibility for antibodies is limited or too slow to restrict viral replication. This is in contrast to antibody-mediated clearance of virus in the central nervous system, as recently reported for alphavirus infection (19). The example of local antibody production in the brain in chronic carriers of lymphocytic choriomeningitis virus may however, suggest that antibody produced in situ may exert effects that are not seen with passive transfer of immune serum (26).

Some mice infected i.v. via the tail vein usually die of VSV, despite the fact that they have generated high titers of neutralizing IgM and IgG antibodies. In addition, mice lacking $CD4^+$ T cells but still able to mount an IgM response are considerably more susceptible to VSV infection than normal animals. These observations suggest that "high-molecular-weight antibodies" of the IgM isotype are less efficient in penetrating tissue or penetrating the blood-brain barrier than antibodies of the IgG isotype. It is interesting that VSV-immune spleen cells (T and B cells) transferred to IFN-α/β $R^{0/0}$

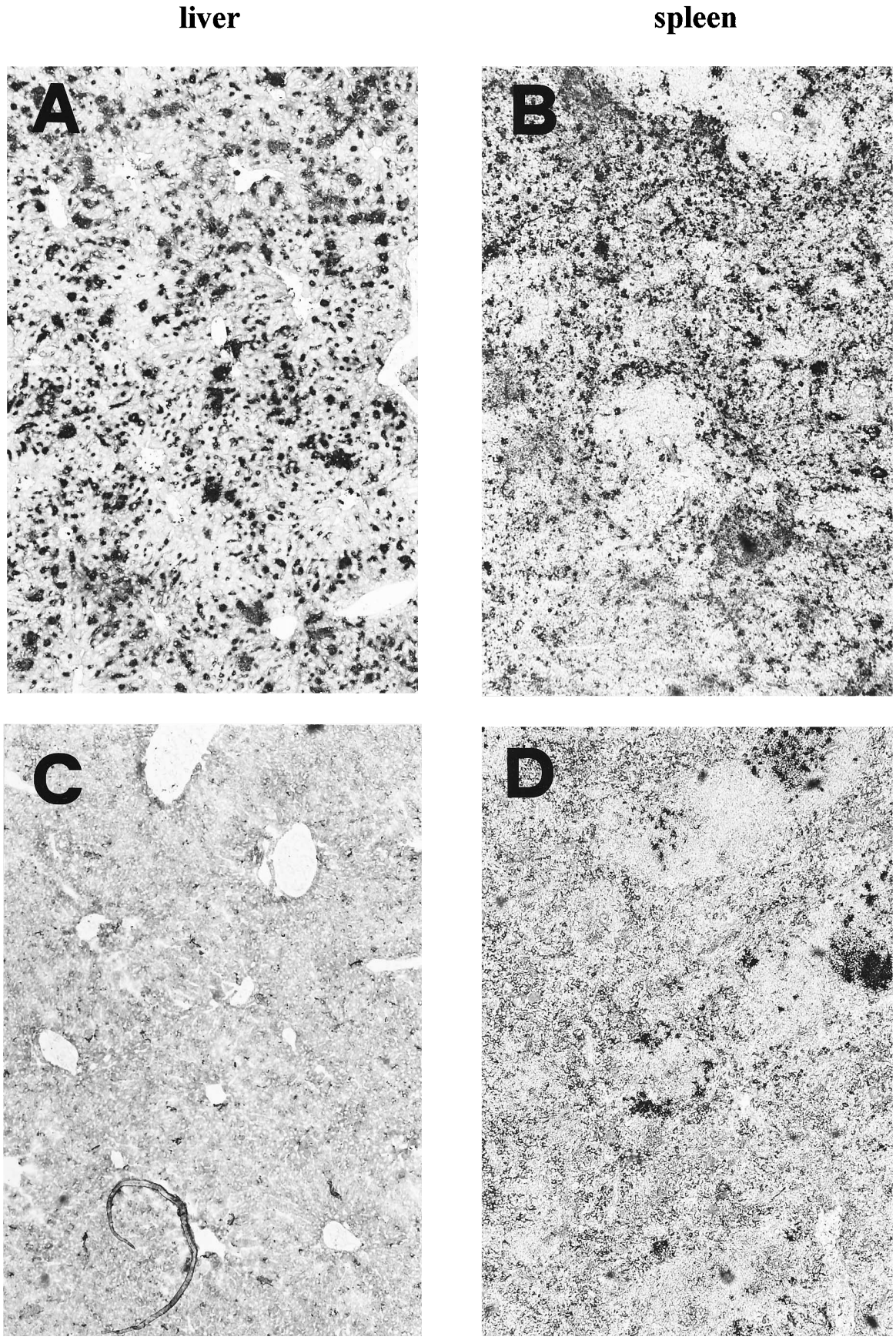

FIG. 2. Immunohistochemical staining of VSV-G in the liver and spleen of i.n.-infected IFN-α/β $R^{0/0}$ mice with or without prior serum reconstitution. IFN-α/β $R^{0/0}$ mice were left untreated (A and B) or transfused with 100 μl of VSV-G-specific immune serum (C and D) 12 h before nasal challenge with 2×10^5 PFU of VSV. Magnification, ×44.

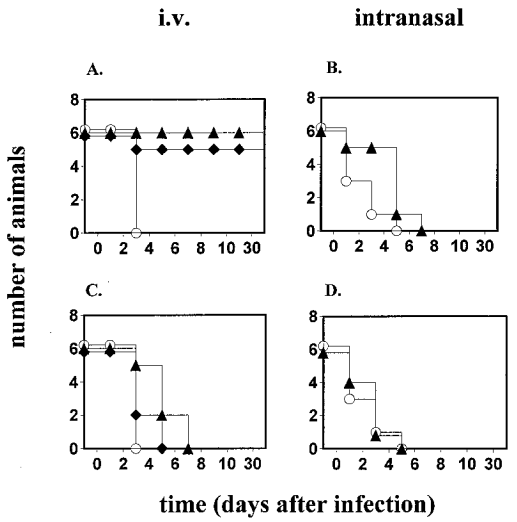

FIG. 3. Passive postexposure immunization of IFN-α/β $R^{0/0}$ mice after systemic (i.v.) or local (i.n.) infection with VSV. Animals were infected i.v. with VSV at 2×10^6 (◆) or 2×10^4 (▲) PFU or challenged i.n. with a dose of 2×10^4 PFU of VSV (▲). Three (A and B) or 16 (C and D) h after infection, 100 μl of VSV-G-specific immune serum was injected i.v., and survival was monitored. Open symbols represent mice that were infected i.v. or i.n. with 2×10^4 PFU of VSV but did not receive immune serum.

mice are too slow and inefficient to generate antibody levels capable of conferring protection in primary infection. Therefore, all these data show that the initial resistance of naive animals against infection with VSV is strictly dependent on the antiviral action of IFN-α/β.

The lack of protection conferred by anti-VSV-N responses is interesting in view of data from the closely related rabies virus model; immunization with the internal nucleoprotein of rabies virus (RV-N) (7, 21, 33) or transfer of anti-RV-N antibodies (20) was able to protect mice from lethal challenge with rabies virus to a certain extent. The underlying mechanism is not understood, and protection by anti-RV-N antibodies seems rather limited. This discrepancy may reflect differences in overall kinetics of virus replication and cytopathic disease, i.e., slow in the case of rabies infection of IFN-α/β $R^{+/+}$ control mice versus more rapid for VSV infection in IFN-α/β $R^{0/0}$ animals. We cannot formally exclude that under conditions of unimpaired natural immunity, VSV-N-specific antibodies may also contribute to protection against VSV. Nevertheless, the results indicate that such a mechanism would be of minor importance.

The results presented confirm that cell-mediated immunity alone cannot protect against VSV. It has been shown that infection with VSV leads to the generation of cytotoxic T lymphocytes, which, depending on the mouse strain, usually recognize VSV-G, VSV-N, or both (15). VSV-specific T lymphocytes were shown to be antivirally active and to control replication of vacc-IND-G in mice (3). Nevertheless, their biological importance in protection or pathogenesis of infection has been rendered unlikely by experiments with $CD8^+$ T-cell-deficient or $CD8^+$ T-cell-depleted mice, which were as resistant to VSV as the controls (8). The role of $CD4^+$ T lymphocytes, other than mediating class switching of B cells, remains unclear. Lymphokines secreted by $CD4^+$ T cells stimulate B cells and recruit macrophages to efficiently phagocytose the viral antigens. Furthermore, switching from the IgM to the IgG isotype is strictly dependent on T helper cells (18). Therefore, VSV-specific $CD4^+$ T cells might be mainly involved in the maintenance of a long-lasting immunity against VSV via local interleukin action but in addition may be involved in the recruitment of B cells into immunologically privileged sites (4, 25, 26).

Passive protection by antibodies against i.n. infection has also been studied with murine hepatitis virus. In this model, neutralizing antibodies protect against fatal encephalitis but do not completely block viral infection of the central nervous system (28).

The mechanism of how antibodies mediate protection against VSV is not fully understood. Lefrancois (16) has shown that VSV-specific antibodies may contribute to protection via both direct neutralization of VSV and Fc-mediated immunity to virus or virus-infected cells (5). Direct neutralization of free virus seems, however, to be the major effector mechanism. This is supported (i) by our finding that VSV-specific monoclonal antibodies with different potentials of complement fixation protected equally well in vivo (data not shown) and (ii) by studies demonstrating that VSV-specific $F(ab')_2$ fragments are sufficient to confer protection in experimental VSV infection (5).

In summary, besides confirming many aspects of anti-VSV immunity, the IFN-α/β $R^{0/0}$ mouse model analyzed here illustrates that under physiological conditions, protective immunity is based on both an immediate innate resistance mediated by type I IFNs and an acquired specific resistance which is largely mediated by neutralizing antibodies. While antibodies protect efficiently against extraneuronal infections, they seem to be poorly protective once VSV has infected brain or nerve tissue.

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